

Transcriptional Induction of the Human Prolactin Gene by cAMP Requires Two cis-Acting Elements and at Least the Pituitary-specific Factor Pit-1*

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To identify the cis-acting elements responsible for cAMP stimulation of human prolactin (hPRL) promoter activity, pituitary GC cells were transfected with 5'-deleted hPRL promoters fused to the chloramphenicol acetyltransferase reporter gene. The proximal regulatory region (coordinates -250 to -42) was sufficient to confer strong cAMP stimulation (\pm 25 fold). Further 5' and 3' deletions performed within this proximal region demonstrated that two types of cis-acting elements are involved in the cAMP regulation: (i) the binding sites of the pituitary-specific factor Pit-1, and (ii) the sequence between coordinates -115 and -85 (named fragment A), which contains a TGACG motif. We show by gel-shift and Southwestern experiments that fragment A binds Pit-1 monomer and also a ubiquitous factor that is neither cAMP-responsive element-binding protein nor activator protein-1. Strong cAMP induction was observed when fragment A was juxtaposed to a Pit-1 binding site. That Pit-1 plays an important role was supported further by the finding that the hPRL proximal region conferred cAMP regulation when linked to the herpes simplex virus thymidine kinase promoter only in pituitary GC cells and not in other heterologous cells, which do not express Pit-1. Furthermore, we observed that concatenated Pit-1 binding sites were able to confer cAMP responsiveness to the thymidine kinase promoter in GC cells.

In eukaryotes, gene transcription is controlled by trans-acting factors that interact with specific DNA sequences in promoter and enhancer elements (reviewed in 1). The prolactin (PRL)¹ gene is a good model for studying both tissue-

specific and hormonally regulated activation of transcription, as its expression is restricted essentially to the pituitary lactotrophic cells and controlled by many hormones. These include, for example, positive and negative hypothalamic factors such as thyrotropin-releasing hormone (2, 3) and dopamine (4, 5), respectively; growth factors such as epidermal growth factor (6, 7); and steroid hormones such as estrogen and glucocorticoids (8, 9).

We reported recently that the pituitary-specific transcription of the human prolactin (hPRL) gene (10) is controlled by three main 5' regulatory regions (11): a proximal region (located between coordinates -250 and -40), a distal region (-1,750 to -1,320), and a superdistal region (-5,000 to -3,500). The first two regions contain several binding sites for the pituitary-specific factor Pit-1 (11, 12). This trans-acting factor (also named GHF1) contains HOMEO and POU domains (13, 14) and confers pituitary-specific expression to both the growth hormone and prolactin (PRL) genes (15, 16); mutations in the endogenous mouse Pit-1 gene lead to a loss of expression of growth hormone and PRL (and also thyroid-stimulating hormone) genes in the pituitary gland, resulting in a dwarf phenotype (17).

Dopamine is one of the most important inhibitory factors of prolactin gene expression. It has been shown that this hypothalamic factor acts at least in part by decreasing the level of cyclic AMP, an intracellular second messenger (4, 18). In line with this proposed mechanism is the observation that when pituitary cells are treated with cAMP analogs or activators of adenylate cyclase, PRL gene transcription is stimulated dramatically within a few min, suggesting that this cAMP stimulation is direct and probably involves post-translational modification of regulatory proteins (4, 19, 20). On the basis of gene transfer experiments, several laboratories have reported that the bovine and rat PRL promoter activities are stimulated by cAMP treatment (21-23). However, the cis-acting elements responsible for the cAMP regulation have not been mapped precisely in these PRL promoters.

To date, two types of cis-acting elements have been shown to confer the cAMP response (24). The first, TGACGTCA, binds the CRE-binding proteins (CREB/activating transcription factor protein family) (25, 26; reviewed in 27). The second, TCCCCANGCG, binds the activator protein-2 (AP-2) (28). It has been shown that the transcriptional activities of both CREB and AP-2 are stimulated via the protein kinase A pathway (28, 29).

In this study we confirm our recent results (30) showing that the activity of the human PRL promoter is stimulated by cAMP although it does not contain any clear consensus sequence for either the CREB or the AP-2 protein. By trans-

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¹ The abbreviations used are: PRL, prolactin; hPRL, human prolactin; AP, activator protein; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; CRE, cyclic AMP-responsive element; CREB, cAMP-responsive element-binding protein; cpt-cAMP, 8-[4-chlorophenylthio]adenosine 3':5'-monophosphate.

fecting pituitary GC cells with a series of 5' and 3' promoter deletion mutants, we determine the cAMP-responsive elements in the hPRL promoter. We then identify by gel-shift and Southwestern experiments a ubiquitous factor, distinct from CREB, which could act in concert with factor Pit-1 to confer cAMP regulation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—All enzyme reactions were performed according to standard procedures (31). Plasmids p5000CAT, p3500CAT, p1750CAT, p1320CAT, p740CAT, p250CAT, p42CAT, and p0CAT which contain sequential 5' deletions of the hPRL promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene, have been described elsewhere (11). Plasmids p164CAT, p138CAT, p115CAT, p95CAT, p69CAT were constructed as follows. 5'-Deleted hPRL promoter DNA fragments were amplified by polymerase chain reactions (PCR). PCR were carried out on the DNA template p250CAT using as 3' primer an 18-mer oligonucleotide whose sequence is a portion of the CAT gene, and as 5' primer oligonucleotides corresponding to different portions of the hPRL proximal promoter and bearing a HindIII site at their 5' ends. The amplified DNA fragments were then digested by HindIII and BglII (the latter cutting 14 base pairs downstream from the hPRL CAP site), gel purified, and inserted in front of the CAT reporter gene by ligation into the HindIII-BglII-digested p740CAT plasmid. The chimeric hPRL-TKCAT constructs were built similarly. Various DNA fragments were amplified by PCR using 5' and 3' primers strategically spaced throughout the hPRL promoter, digested by HindIII and XbaI, gel purified, and inserted in front of the thymidine kinase promoter by ligation into the HindIII-XbaI-digested pBLCAT2 plasmid (32). Construction of the P1-TKCAT, P2-TKCAT, P2 mut-TKCAT and CRE-TKCAT plasmids was performed by synthesizing and annealing the corresponding sense and antisense oligonucleotides (see their sequences below) and cloning them in the pBLCAT2 plasmid.

The correct sequence of all these constructs was confirmed by dideoxy chain termination sequencing (33).

All plasmids were prepared by alkaline lysis, purified by centrifugation in CsCl-ethidium gradients, treated with RNase, centrifuged through a 1 M NaCl cushion, extracted with phenol-chloroform, and finally precipitated with ethanol. The concentrations of all plasmid preparations were determined by measuring the absorbance at 260 nm.

Oligonucleotides—The double-stranded oligodeoxyribonucleotides used in plasmid constructions or in gel-shift assays were obtained from Eurogentec (Liège, Belgium), except for fragment A synthesized by PCR. The AP-1 oligonucleotide contains the AP-1 site of the human collagenase promoter (34). The CRE oligonucleotide contains the CREB binding site of the human chorionic gonadotropin α -subunit promoter (35). For each oligonucleotide, the sequence of one strand is presented below.

P1: CTAGAATGCCTGAATCATTATATTCATGAAGATATC

P2: GATCCCTTCTGAATGAATAAGAAATAAAAAG

P2mut: GATCCCTTCTGTAATACAAATAAGAAATAAAAAG

AP-1: AGCTTAAAGCATGAGTCAGACACCT

CRE: CTAGAAATTGACGTCATGGTAA

fragment A: CCAAGCTTGGGGGTAATCTCAATGACGGAAA-TAGATGATCTAGACG

Cell Cultures and Electroporation—GC cells (derived from a rat pituitary tumor), BeWo and JEG-3 cells (both derived from a human placental choriocarcinoma) were grown in monolayer in Ham's F-12 supplemented with 10% fetal calf serum. For the transfection experiments, cells were harvested with trypsin-EDTA and resuspended in Ham's F-2 plus 10% fetal calf serum at a concentration of 40×10^6 cells/ml for GC cells, 20×10^6 cells/ml for JEG-3 cells, or 7.5×10^6 cells/ml for BeWo cells. Purified plasmid DNA ($40 \mu\text{g}$ of hPRL 5' deletion mutant plasmid and $15 \mu\text{g}$ of the chimeric hPRL-TKCAT constructs) was mixed with 32×10^6 GC cells, 16×10^6 JEG-3 cells, or 6×10^6 for BeWo cells. Cells were then exposed to a single pulse of 250 V/4 mm and 1,500 μF capacitance for GC cells; 200 V/4 mm and 1,050 μF capacitance for JEG-3 and BeWo cells, using a "Cellject" apparatus (Equibio, Liège, Belgium). The transfected cells were transferred to six tissue-culture dishes (35 mm). After 3 h for GC cells and 16 h for JEG-3 and BeWo cells, half of the transfected cells were incubated for about 16 h with 500 μM cpt-cAMP (Boehringer Mannheim), a nonmetabolizable cAMP analog. The cells were then harvested by scraping, washed in phosphate-buffered saline, resuspended

in 100 μl of 250 mM Tris/HCl (pH 7.6), and frozen at -70°C .

CAT Assay—Transfected cells were disrupted by three cycles of freezing/thawing with sonication and the extracts centrifuged for 15 min at $12,000 \times g$ in an Eppendorf centrifuge to remove the debris. The cell extract supernatants were incubated for 6 min at 65°C and centrifuged for 15 min at $12,000 \times g$. Then, a mixture containing 10 μl of [^{14}C]chloramphenicol (25 $\mu\text{Ci}/\text{ml}$; Amersham Corp.), 22 μl of acetyl coenzyme A (10 mM, Sigma), and 100 μl of 125 mM Tris/HCl (pH 7.6) was added to the supernatants. The reactions were stopped by addition of ethyl acetate. After extraction, chloramphenicol and its acetylated forms were separated by thin layer chromatography (36). After autoradiography, the spots containing the acetylated products and the remaining substrate were cut from the thin layer chromatography plates, scraped into separate scintillation vials, and counted.

Preparation of Extracts—Nuclear (for GC cells and rat liver) and whole-cell (for HeLa, BeWo, and IM9 cells) extracts were prepared as described by Dignam *et al.* (37) and Manley (38), respectively. Protein concentrations in these extracts were determined by the Bradford assay (52) with bovine serum albumin as a standard.

Gel-shift and Southwestern Assay—DNA fragments for gel-shift and Southwestern analyses were either synthetic double-stranded oligonucleotides (P1 and P2 fragments) labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase or fragment A generated and labeled by PCR in the presence of [α - ^{32}P]dCTP (see above for the sequence of these DNA fragments).

Gel-shift analyses were performed with 2.5 μg of nuclear protein extract or 7 μg of whole-cell extract, 1 μg of poly(dI-dC), and 10,000–15,000 cpm of ^{32}P -labeled DNA fragments in 20 mM Tris/HCl (pH 7.9), 5 mM MgCl_2 , 50 mM KCl, 0.1 mM EDTA, 10% glycerol. The resulting protein-DNA complexes were resolved by electrophoresis on a prerun 5% polyacrylamide gel with $0.25 \times \text{TBE}$ as running buffer.

Southwestern analyses were performed on 50 μg of GC nuclear extract or 100 μg of BeWo whole-cell extract that had been subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and electroblotted onto 0.2- μm nitrocellulose filters. The filter-bound proteins were denatured/renatured using 6 M guanidine HCl and probed with labeled and concatenated DNA fragment A as described by Vinson *et al.* (39).

RESULTS

The Essential cAMP-responsive Element of the hPRL Promoter Is Confined within the First 250 Base Pairs—To study cAMP regulation of the hPRL promoter, a set of constructs containing various 5' deletions of the hPRL promoter fused to the CAT reporter gene were introduced by electroporation into pituitary GC cells. Transfected cells were then incubated for 16 h with or without cpt-cAMP, a nonmetabolizable cAMP analog that activates cAMP-dependent protein kinase. CAT enzymatic activities were determined in the transfected cell extracts, and the cAMP induction ratios (induced *versus* noninduced CAT expression) were calculated for each construct. As shown in Fig. 1, plasmids with promoter end points $-5,000$, $-3,500$, $-1,750$, $-1,330$, -740 , and -250 clearly respond to cpt-cAMP (about 20–25-fold stimulation). Removal of sequences from -250 to -42 causes the cAMP stimulation to drop drastically, from 25-fold to 3-fold. The p42CAT construct contains only the TATA box and is in fact stimulated to the same extent as the two negative controls: the HSV thymidine kinase and Rous sarcoma virus promoters. This 3-fold stimulation is considered nonspecific and has been observed by others (22, 41–43). Thus, an essential cAMP-responsive region appears to lie between coordinates -250 and -42 , within the proximal regulatory region of the hPRL promoter.

Determination of the 5' and 3' Boundaries of the cAMP-responsive Elements—To locate more precisely the 5' boundary of the sequences required for cAMP stimulation we constructed a set of 5' deletion mutants within the proximal region (see "Experimental Procedures"). All of these mutated hPRL promoters fused to the CAT reporter gene were intro-

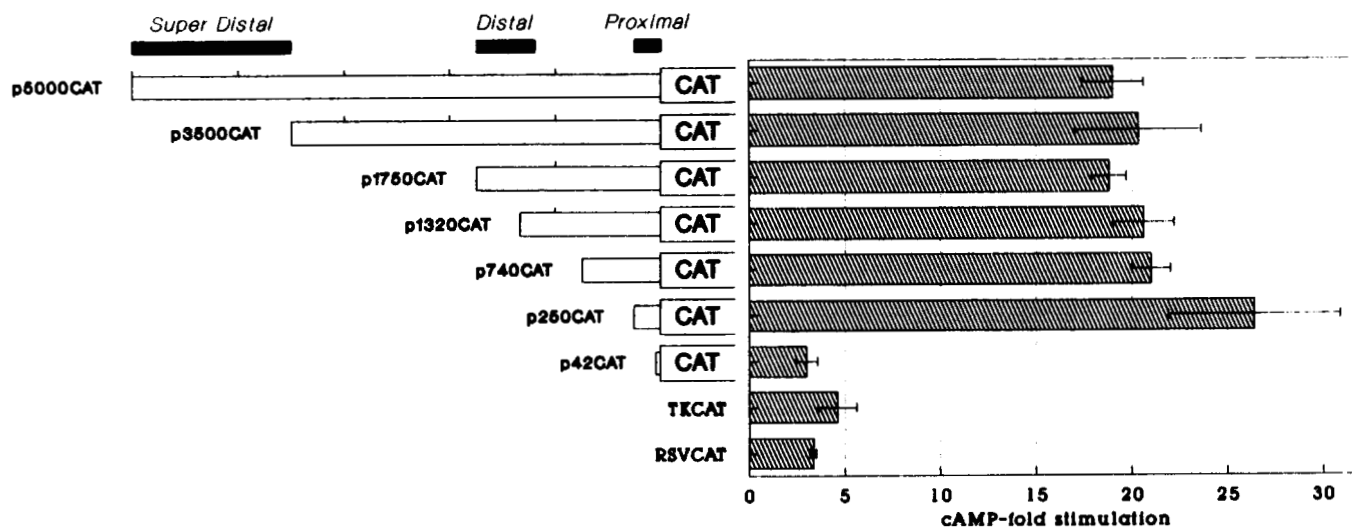


FIG. 1. cAMP induction of hPRLCAT 5' deletion mutants in GC cells. GC cells were transfected with the hPRLCAT constructs as described under "Experimental Procedures," and 3–4 h later half of the transfected cells were incubated with 500 μ M cpt-cAMP. CAT activity was determined after about 16 h, and the stimulation was calculated as the ratio of CAT activities in cAMP-treated versus untreated cells. Values are the means \pm S.E. of two independent transfection experiments performed in triplicate. Black boxes indicate the location of the proximal, distal, and superdistal regulatory regions in the hPRL promoter (11).

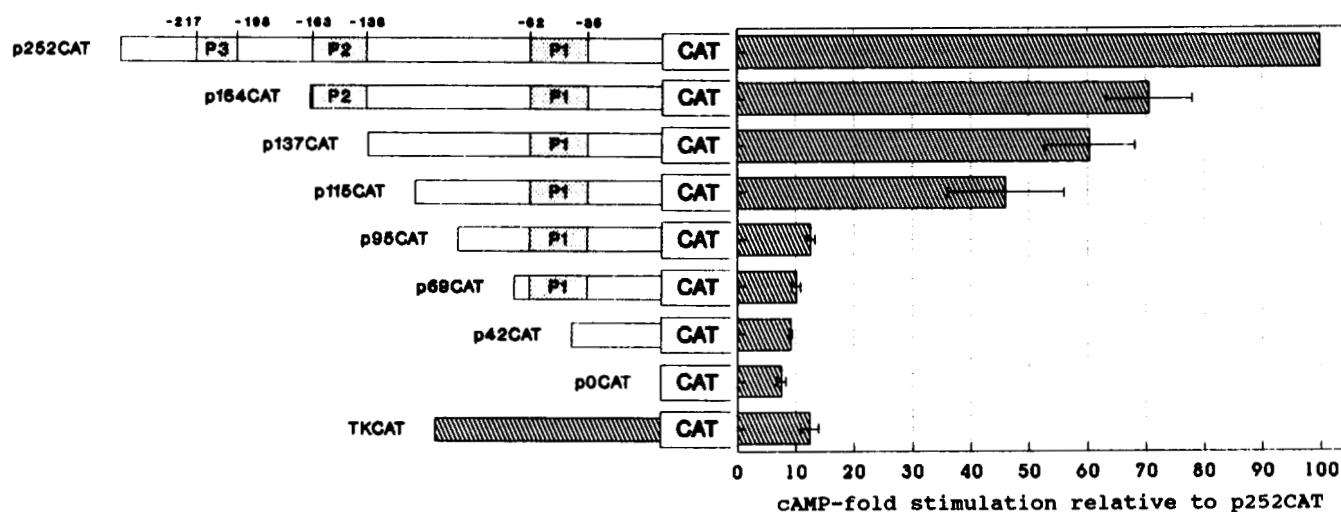


FIG. 2. cAMP induction of proximal hPRLCAT 5' deletion mutants in GC cells. GC cells were transfected with 40 μ g of each hPRLCAT construct and were treated as described in Fig. 1. As the extent of cAMP induction varied in the different transfection experiments, the stimulation obtained with each construct was normalized with that obtained for the p250CAT construct (whose cAMP stimulation average was 33-fold \pm 7 in these six transfection experiments). Values are means \pm S.E. of six independent transfection experiments performed in triplicate. The three Pit-1 binding sites in the hPRL proximal region are indicated by boxes P1, P2, and P3 (12).

duced into GC cells and tested for their cAMP responsiveness. As the extent of cAMP induction varied somewhat in the different experiments, the cAMP stimulation ratio obtained for each construct was expressed as a percentage of that obtained with the p250CAT construct within each experiment. Fig. 2 shows that as increasingly larger portions of the promoter were deleted, cAMP induction decreased. The relative stimulation dropped gradually to about half its value with deletions in the -250 to -115 region, which contains two high affinity Pit-1 binding sites (P3 and P2). Additional deletion of the sequence from -115 to -95 caused a further, steep drop. In fact, no specific stimulation was observed with the p95CAT, p69CAT, and p42CAT constructs. Since all constructs exhibited, under our conditions, a basal activity above

the background level (*i.e.* the CAT activity obtained with the promoterless p0CAT construct), we can conclude that the differences in stimulation between these constructs indeed reflect differences in cAMP responsiveness.

To determine the 3' boundary of the cAMP-responsive elements, various fragments of hPRL promoter were cloned upstream from the thymidine kinase promoter (see "Experimental Procedures"), and their ability to confer cAMP stimulation was tested in GC cells. We used as a positive control the plasmid p(CRE)1 \times TKCAT, which contains one copy of the palindromic CRE sequence (derived from the human chorionic gonadotropin α -subunit gene; 35) in front of the thymidine kinase promoter. Results, presented in Fig. 3, are expressed in this case with respect to the stimulation ratio

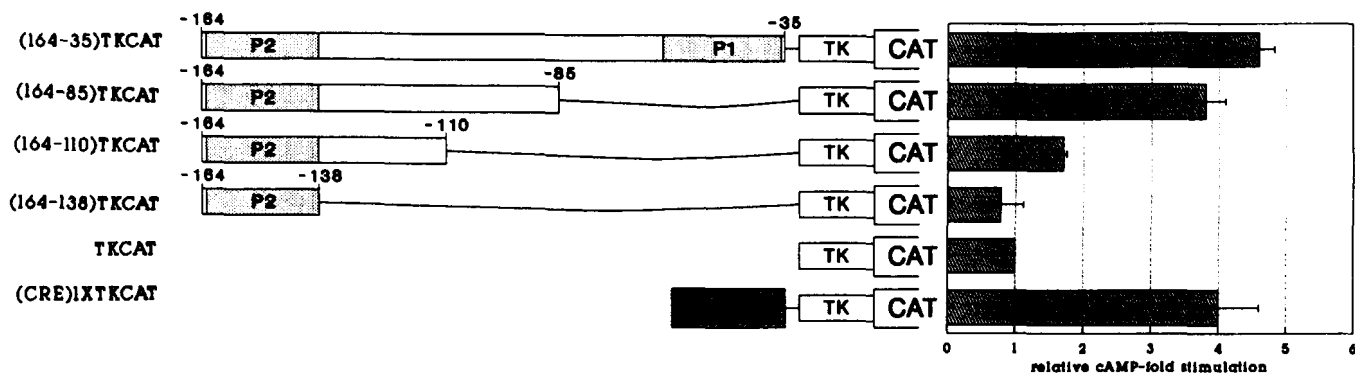


FIG. 3. cAMP induction of the hPRL-TKCAT 3' deletion mutants in GC cells. Cells were electroporated with 15 μ g of each chimeric hPRL-TKCAT construct and treated as described in Fig. 1. cAMP induction obtained with each construct was normalized with that obtained for the negative control TKCAT (which is nonspecifically stimulated 4-fold \pm 0.9). Values are means \pm S.E. of three independent transfection experiments performed in triplicate.

observed for the TKCAT construct (4-fold \pm 0.9). Fragment (-164 to -35) was able to confer to the thymidine kinase promoter a cAMP response as strong as that of the positive control CRE. Deletion of the sequence between -35 and -85 slightly diminished cAMP stimulation (about 1.2-fold diminution) whereas the major drop (about 2.2-fold) occurred with further deletion of the sequence from -85 to -110. This confirms the importance of the latter region, already observed with the 5' deletions. Complete loss of stimulation occurred with additional deletion of the sequence from -110 to -138.

The combined results obtained with 5' and 3' deletion mutants lead to two main conclusions. (i) The removal of Pit-1 binding sites correlates with decreased cAMP stimulation; this trans-acting factor seems thus to play a role in hPRL promoter regulation by cAMP. (ii) The major loss of cAMP stimulation is observed when the sequence from -85 to -115 is deleted; this strongly suggests that an important cAMP-responsive element is located in this sequence.

Several Pit-1 Binding Sites Confer the cAMP Response to a Heterologous Promoter in GC Cells—To determine whether any sequences of the proximal regulatory region are able to confer cAMP responsiveness, various DNA fragments of the hPRL promoter were inserted in one or several copies in front of the thymidine kinase promoter. These chimeric hPRL-TKCAT constructs were introduced into GC cells and tested as described above. All of these constructs were compared with the two positive controls p(CRE)1xTKCAT and p(CRE)2xTKCAT as to their cAMP responsiveness. Fig. 4 shows that when inserted in one copy, no fragment conferred cAMP stimulation. When inserted in several copies, both Pit-1 binding sites (either P1 or P2) gave significant cAMP induction. By contrast, three copies of a mutated P2 site, containing two base substitutions in the Pit-1 consensus motif (see "Experimental Procedures") and having a much lower affinity for this factor, did not respond at all to cAMP treatment. This result strengthens the view that Pit-1 is involved in cAMP regulation of the hPRL promoter. When fragment A (comprising sequences from -115 to -85) was inserted in two copies, a small (1.7-fold) cAMP induction was also observed. No response was observed with three copies of fragment B (from -138 to -115). It is interesting to note at this point that although one copy of either fragment A or P2 gave no cAMP stimulation, they produced a strong response when juxtaposed (plasmid p(164-85)TKCAT in Fig. 3).

The cAMP-responsive Elements of the hPRL Gene Are Active Only in Pituitary Cells—To investigate further whether the presence of the pituitary Pit-1 factor is absolutely neces-

sary for cAMP induction of hPRL promoter activity we performed the same transfection experiments in two placental cell lines derived from human choriocarcinoma (JEG-3 and BeWo), which are devoid of this trans-acting factor.² As the hPRL promoter is inactive in these cells and as no detectable CAT activity is obtained with p250CAT, we transfected the cells with the chimeric hPRL-TKCAT constructs only. As shown in Table I, the hPRL proximal region (-164 to -35) was unable to confer any cAMP stimulation to the thymidine kinase promoter in JEG-3 and BeWo cells, in contrast to the strong cAMP response obtained in GC cells. This was not caused by an inability of these cells to respond to cAMP treatment, since the positive control p(CRE)2xTKCAT was highly stimulated. These results strongly suggest that the presence of Pit-1 is crucial to obtaining a full response of the hPRL promoter to cAMP stimulation. Interestingly, the weak cAMP response obtained in GC cells with the p(115-85)2xTKCAT construct was also observed in JEG-3 and BeWo cells.

Binding of trans-Acting Factors to the hPRL Proximal Promoter Is Not Influenced by cAMP Treatment—As the transfection experiments described above show that the Pit-1 binding sites and the sequence from -115 to -85 are involved in cAMP regulation, gel-shift assays were performed to determine whether (i) Pit-1 binding is affected by cAMP treatment, and (ii) the sequence from -115 to -85 (fragment A) does bind a trans-acting factor. Nuclear extracts were prepared from GC cells treated or not for 1 h with 10 μ M forskolin, an activator of adenylate cyclase.

Increasing concentrations of the treated or untreated extract were incubated with labeled Pit-1 binding site P1 (sequence from -67 to -35). Fig. 5A shows the results for untreated extract. Two major complexes appeared: the C1 complex (actually a doublet band) and the slower migrating C2 complex. The latter's constant appearance at higher protein concentrations suggests that C2 represents a Pit-1 dimer and C1 a Pit-1 monomer. Ingraham *et al.* (44) have also recently observed two such complexes and demonstrated that they indeed correspond with the monomeric and dimeric forms. Fig. 5B shows that unlabeled P1 fragment readily displaced labeled P1 in the C2 complex (which disappeared at a 10-fold molar excess of unlabeled P1) whereas disappearance of the C1 band required a 200-fold molar excess of unlabeled P1 fragment. This clearly shows that the monomeric complex is much more difficult to displace with the unlabeled fragment than the dimeric complex. Fig. 5, C and

² B. Peers, unpublished data.

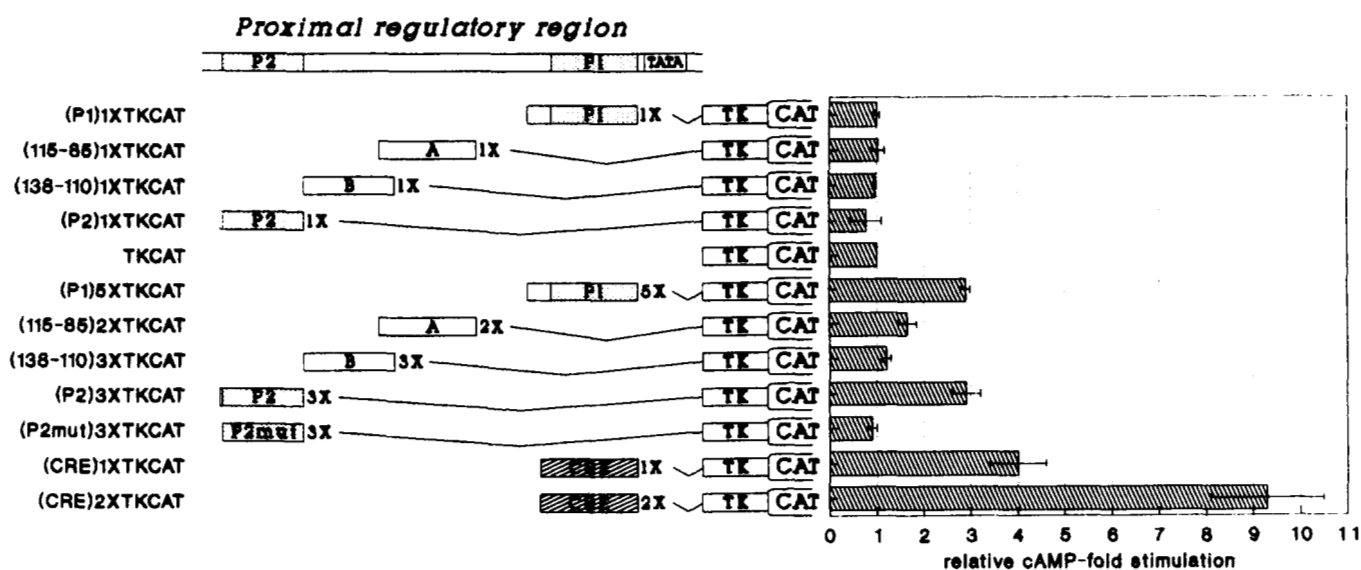


FIG. 4. Ability of various hPRL proximal sequences to confer cAMP responsiveness to the thymidine kinase promoter in GC cells. Cells were electroporated with 15 μ g of each construct and treated as described in Fig. 3. Values are means of two to four independent transfection experiments performed in triplicate.

TABLE I

Comparison of cAMP response of the chimeric hPRL-TKCAT constructs in JEG-3, BeWo, and GC cells

Cells were transfected as described under "Experimental Procedures." Induction is expressed relative to the TKCAT construct (which is nonspecifically stimulated 1.2-fold \pm 0.1 in JEG-3, 2-fold \pm 0.3 in BeWo, and 4-fold \pm 0.9 in GC cells). Values are means \pm S.E. of at least two independent transfection experiments performed in triplicate.

Plasmid	Relative cAMP stimulation		
	JEG 3	BeWo	GC
(164-35) 1XTKCAT	0.9 \pm 0.1	1 \pm 0.5	4.6 \pm 0.3
(115-85) 2XTKCAT	2.0 \pm 0.4	1.5 \pm 0.6	1.7 \pm 0.3
(CRE) 2XTKCAT	13 \pm 4	13 \pm 2	9.3 \pm 1.5
TKCAT	1	1	1

D, shows that forskolin treatment did not alter binding of monomeric or dimeric Pit-1 to either the P1 or the P2 sites (compare the first and second lanes in Fig. 5, C and D).

Fig. 5E shows the results of experiments in which labeled fragment A (–115 to –85) was incubated with GC nuclear extract (2.5 μ g). Here again, two complexes were detected: Ca, a doublet, and Cb. In the presence of a 100-fold molar excess of unlabeled fragment A, complex Cb disappeared, suggesting that the corresponding protein specifically binds to this fragment with a high affinity. Complex Ca, on the other hand, was only attenuated. In competition experiments with other oligonucleotides binding the CREB, AP-1, or Pit-1 factors, complex Cb was not affected whereas the intensity of the Ca doublet decreased with 100-fold molar excess of the Pit-1 binding sites P1 and P2 (in fact, Ca completely disappeared with a 500-fold molar excess of P1, P2, or fragment A; data not shown). To determine whether the proteins binding to fragment A are ubiquitous and affected by cAMP treatment, extracts from heterologous cells and forskolin-treated GC cells were tested. Fig. 5F shows that the Cb complex was obtained with all extracts tested whereas the Ca complex is pituitary specific. No change in binding activity could be detected with cAMP treatment. These results suggest two conclusions. (i) High affinity binding occurs between the (–115 to –85) sequence and a ubiquitous factor that is neither CREB nor

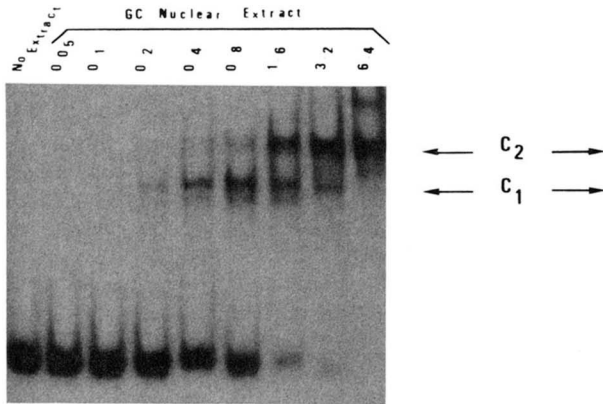
AP-1. (ii) The same sequence also binds Pit-1, but the affinity is lower (a monomeric form of Pit-1 is probably involved, as it takes a large amount of competing Pit-1 binding sites to displace Ca).

To test these two hypotheses further, Southwestern experiments were performed. GC and BeWo protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted on a nitrocellulose filter, and incubated with labeled fragment A. The results (Fig. 6) reveal that fragment A binds a 43-kDa protein present in both extracts. With the GC extract, we further observed (i) a slight 31–33-kDa doublet which is likely to represent the two described forms of Pit-1 (13, 14), and (ii) additional bands at 68 and 90 kDa whose significance is yet unclear. The Southwestern and gel-shift data thus both indicate that fragment A binds a ubiquitous factor (which would have an apparent molecular mass of 43 kDa) and pituitary-specific factors (notably Pit-1 monomer).

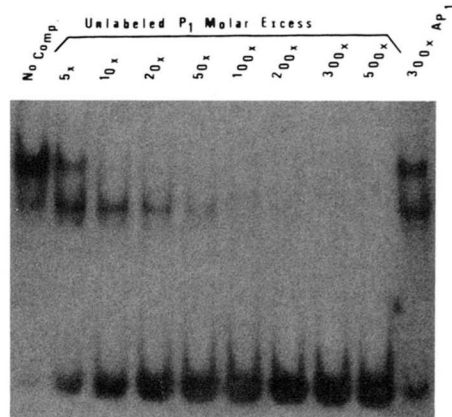
DISCUSSION

The aim of this study was to identify the cAMP-responsive elements of the hPRL gene. Our results show that the proximal regulatory region of the hPRL promoter, comprising the first 250 base pairs, is sufficient to give full cAMP stimulation. To locate the cAMP-responsive elements more precisely, 5' and 3' deletion mutants were generated within this proximal region as several chimeric hPRL-TKCAT constructs. The results obtained by transfecting pituitary GC cells and heterologous cells with these constructs led to two conclusions. First, the pituitary-specific factor Pit-1 does play an important role in cAMP regulation. Four observations support this view. (i) Successive removal of the Pit-1 binding sites in the 5' or 3' deletions progressively reduces cAMP stimulation. (ii) Several copies of either the P1 or P2 Pit-1 binding sites confer cAMP responsiveness to the thymidine kinase promoter. (iii) An analogous construct having reduced affinity for the Pit-1 protein, because of two base substitutions in the Pit-1 consensus binding motif, produces no cAMP stimulation. (iv) The cAMP response of the chimeric hPRL-TKCAT constructs was observed only in pituitary cells and not in heterologous cell lines lacking the Pit-1 factor.

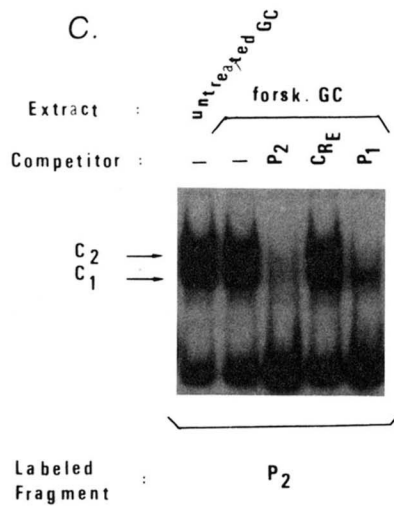
A.



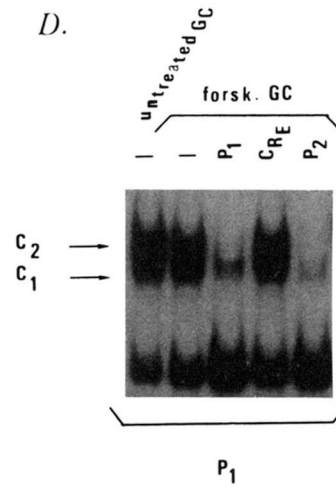
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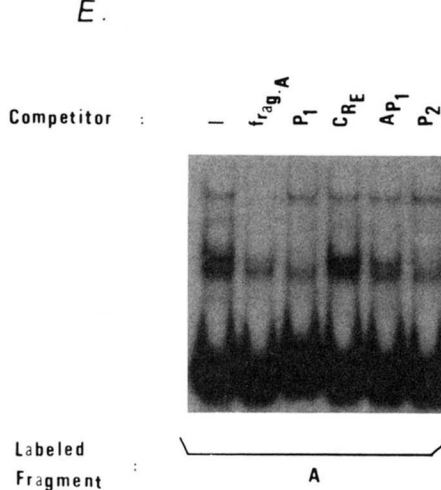
C.



D.



E.



F.

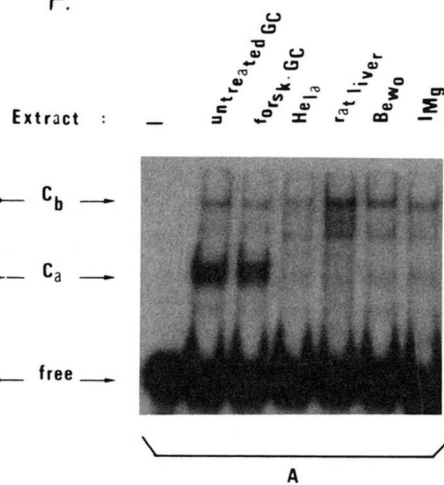


FIG. 5. Binding of Pit-1 and a ubiquitous factor to the hPRL proximal region is not affected by cAMP treatment. Gel-shift assays were performed as described under "Experimental Procedures." The labeled DNA fragments used in the assays are indicated below each panel. In the competition assays (panels B, C, D, and E) the labeled fragment was incubated with 2.5 μ g of GC nuclear protein in the presence of 100-fold molar excess (25 ng) of unlabeled double-stranded oligonucleotide (except when noted: panel B). The sequence of these oligonucleotides is presented under "Experimental Procedures."

At this point it is interesting to note the previous observation, made by Dana and Karin (40), that the activity of the human growth hormone promoter (which also binds Pit-1) is stimulated by cAMP in pituitary cells only and not in heterologous cells. This supports the notion that factor Pit-1 is involved not only in tissue specificity but also in regulation by cAMP.

Our second major conclusion is that the sequence between coordinates -115 and -85 (named fragment A) is also involved in cAMP regulation, as seen by the major loss of cAMP response in both 5' and 3' deletions of this sequence. Our gel-shift assays demonstrate that fragment A is recognized by the Pit-1 monomeric protein and also that this DNA fragment specifically binds a ubiquitous factor which, according to the Southwestern experiments, has an apparent molecular mass of 43 kDa. The Southwestern blot also indicates the presence of 68- and 90-kDa proteins in GC nuclear extract binding fragment A. Further experiments are required to determine which proteins mediate the effect of fragment A. Nevertheless, the functional importance of fragment A is further supported by the fact that it is highly conserved in the rat, bovine, and human PRL genes (see Fig. 7). Although this sequence contains the TGACG motif (see Fig. 7), similar to the classical CRE (24, 25, 27), competition experiments show that the ubiquitous factor binding fragment A is not CREB. When inserted in two copies in front of the thymidine kinase promoter, fragment A confers some cAMP induction, albeit weak as compared with the classical CRE palindrome. Experiments are under way to determine whether more numerous copies might increase induction.

Taken together, our results indicate that the cAMP-responsive region of the hPRL gene is in fact composed of several

elements that do not individually respond to cAMP, but when juxtaposed, give strong stimulation. This suggests cooperativity between the different cis-acting elements of the hPRL proximal region. Cooperativity between tissue-specific and cAMP-responsive elements has been described previously for other genes, as for example the genes encoding tyrosine aminotransferase (45) and α -subunit glycoprotein (35, 46).

Iverson *et al.* (47) have recently constructed a series of linker scanning mutations in the proximal regulatory region of the rat PRL gene. Their results agree with ours in that mutations within each Pit-1 binding site reduce cAMP stimulation of rat PRL promoter activity. Furthermore, they observed that a mutation in the -97 to -84 region (corresponding with fragment A in the human PRL promoter; see Fig. 7) affects cAMP response. As this region contains the TGACG motif, these authors proposed that the CREB protein might bind this sequence. Our gel-shift assays indicate otherwise, since none of the factors binding fragment A is displaced by an excess of unlabeled CRE oligonucleotide. Iverson *et al.* (47) also showed that mutations in the rat PRL promoter between coordinates -78 and -71 affect the cAMP response. As this region contains the GGGAGGGG sequence similar to the AP-2 binding consensus motif, they suspected this factor to be involved in cAMP regulation. Our results suggest, however, that in the human PRL promoter this region is not important for cAMP regulation. Two arguments support this view. One is that the p95CAT construct, containing most of this region, does not respond to cAMP. The second is that 3' deletion of sequences from -35 to -85 does not substantially affect cAMP regulation. This difference between the human and rat PRL promoters might arise from the fact that the (-65 to -85) sequence is less conserved and that the GGGAGGGG motif is not present in the human promoter (see Fig. 7).

One interesting and surprising observation in our study is that several Pit-1 binding sites confer cAMP responsiveness. McCormick *et al.* (48) have recently shown that the Pit-1 gene promoter contains two CREB binding sites and that its activity is stimulated by cAMP treatment. This could suggest that the cAMP response conferred by Pit-1 binding sites is indirect and is caused by an increased Pit-1 protein concentration during cAMP treatment. However, several laboratories have proven that PRL gene transcription is directly stimulated within the first few min of cAMP treatment (19, 20). It is thus plausible that both direct and indirect effects may contribute to cAMP regulation of PRL expression. Analysis of the Pit-1 amino acid sequence reveals, between the N-terminal transactivation domain and the POU domain, the sequence LRRKSK, similar to the consensus sequence XRRXSX (where X is any amino acid) recognized by protein kinase A (49). This suggests that Pit-1 activity could be regulated by phosphorylation. As our gel-shift assays do not

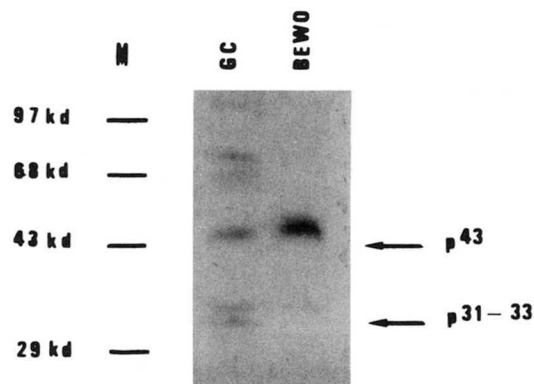


FIG. 6. Southwestern blots of GC (first lane) and BeWo (second lane) protein extracts. The assay was performed as described under "Experimental Procedures," using as probe the 32 P-labeled fragment A. Molecular weight markers are shown on the left. The 43-kDa ubiquitous protein and the pituitary-specific 31-33 kDa doublet are indicated by arrows on the right.

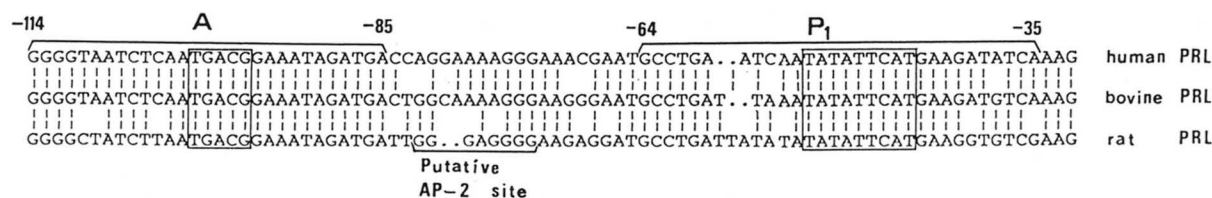


FIG. 7. Sequence comparison between the human, bovine, and rat PRL proximal promoter regions. Sequences between coordinates -32 and -114 of the human (upper line), bovine (middle line), and rat (lower line) PRL genes were aligned, and gaps were introduced to maximize similarity using the "GAP" program from the University of Wisconsin Genetic Computer Group. Locations of fragment A (with its TGACG motif) and of the first Pit-1 binding site P1 (with its consensus motif A/TA/TTATNCAT) are indicated by boxes. The putative AP-2 binding motif in the rat PRL promoter is also shown (24, 47).

show any change in Pit-1 binding after cAMP treatment it seems that if Pit-1 activity is controlled directly, this does not occur at the binding level but rather at the level of transcriptional activation. In this respect it is interesting to note that the N-terminal region of factor Oct-2 (which also contains POU and HOMEODOMAINS domains) is phosphorylated and that phosphorylation controls transcriptional activation rather than binding to DNA (50). Similar conclusions have been drawn for the CREB protein; protein kinase A phosphorylates CREB on serine 133, increasing its ability to activate transcription (51). We are currently checking whether the activity of Pit-1 can be controlled directly by phosphorylation.

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